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PCV2d-2 is the predominant type of PCV2 DNA in pig samples collected in the U.S. during 2014-2016

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Highlights

- Porcine circovirus type 2 (PCV2) is an important pig virus.
- PCV2 vaccination is widely used.
- PCV2d-2 is the main PCV2 genotype in US samples.

Abstract

Porcine circovirus type 2 (PCV2) vaccination was introduced in the US in 2006 and since has been adopted by most pig producers. While porcine circovirus associated disease (PCVAD) outbreaks are now relatively uncommon in the US, PCV2 remains a concern which is emphasized by increasing numbers of PCR and sequencing requests for PCV2. In the present study, randomly selected lung tissues from 586 pigs submitted in 2015 were tested for presence of PCV2 DNA. Positive samples were further characterized by sequencing and combined with available PCV2 open-reading-frame (ORF) 2 sequences from the client data base of the Iowa State University Veterinary Diagnostic Laboratory. The prevalence of PCV2 in the randomly selected lung tissues was 23% (135/586) with 11.3% PCV2a, 29% PCV2b and 71.8% for PCV2d subgroup PCV2d-2. A total of 455 ORF2 sequences obtained from 2014 through 2016 were analyzed and PCV2d accounted for 66.7% of the 2014 sequences, 71.8% of the 2015 sequences, and 72% of the 2016 sequences. Interestingly, only 1.9% (9/455) of the sequences belonged to the recently identified PCV2e genotype. The present data indicates that despite an almost 100% PCV2 vaccine coverage in the US, PCV2 DNA can still be detected in almost 1 of 4 randomly selected pig tissues. PCV2d-2 is now the predominant genotype in the USA suggesting that PCV2d-2 may have some advantage over PCV2a and PCV2b in its ability to replicate in pigs under vaccination pressure.

1. Introduction

Porcine circovirus (PCV) type 2 is considered to be among the most important pathogens in the global swine industry (Allan et al., 2012; Meng, 2012; Segalés, 2015). PCV2 was identified in 1998 in pigs from Canada with postweaning multisystemic wasting syndrome (PMWS) (Ellis et al., 1998; Segalés et al., 2005). At least five PCV2 genotypes including PCV2a, PCV2b, PCV2c, PCV2d and the recently identified PCV2e have been recognized or are proposed to date (Davies et al., 2016; Franzo et al., 2015b; Xiao et al., 2015).

The first identified PCV2 genotype, PCV2a, is also the oldest genotype and most currently available commercial PCV2 vaccines are based on this virus cluster. PCV2a predominated in the global pig herds until around 2003 when a global genotype shift from PCV2a to PCV2b was observed (Beach and Meng, 2012). This genotype shift from PCV2a towards PCV2b was often associated with apparent increased severity of clinical disease and high morbidity and mortality in pig herds located in regions where PCV2b was introduced (Beach and Meng, 2012; Patterson and Opriessnig, 2010). Since its appearance in North America in 2005, PCV2b has been the predominant strain in the US pig population.

PCV2c was discovered in archived swine serum samples in Denmark (Dupont et al., 2008) and recently was also detected in a feral pig in Brazil (Franzo et al., 2015a) but is likely of minor importance. PCV2d can be classified into PCV2d-1 and PCV2d-2, with substantial genetic divergence between them (Xiao et al., 2015). PCV2d-1 strains were first identified in 2002 in China (Cortey et al., 2011; Wang et

al., 2009) while PCV2d-2 strains were first recognized in 2008 in China and have been linked with increased virulence (Guo et al., 2010, 2012; Xiao et al., 2015). In 2012, PCV2d-2 was also identified in US pigs in cases of apparent vaccine failure (Xiao et al., 2012, 2015). Subsequently, PCV2d-2 has also been detected in South America, Europe and Asia (Segalés, 2015; Xiao et al., 2015).

In 2015, four new and distinct PCV2 sequences were identified in pig samples from Mexico and the US (Harmon et al., 2015). Ten similar PCV2 sequences were also identified in the US in a recent retrospective analysis with the earliest sequence dating back to 2006 (Davies et al., 2016). The open-reading-frame (ORF) 2 of these PCV2 isolates is 717 bp in length, 15 bp longer than that of PCV2a or PCV2b isolates and 12 bp longer than that of PCV2c and PCV2d, and shows only about 85% identity to other known PCV2 ORF2 sequences (Davies et al., 2016; Harmon et al., 2015). Due to the substantial genomic differences of the ORF2 composition of this group of PCV2 isolates, a new PCV2 genotype, PCV2e, has been proposed (Davies et al., 2016). Little is known to date on distribution, prevalence rates and importance of PCV2e isolates.

The objectives of this study were to investigate the prevalence of PCV2 DNA in a set of 586 randomly selected 2015 diagnostic pig lung submissions and to determine the distribution of PCV2 genotypes in US pigs prior to April 2016.

2. Material and methods

2.1. Tissue sample source and origin, processing and DNA extraction

2.1.1. Sample source and origin. A total of 586 lung tissues were randomly selected from routine diagnostic cases submitted from January through March 2015 to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). The lung samples originated from 22 U.S. states (Table 1) and pig age ranged from 2 days to adults. Overall there were 79/586 suckling pigs (up to 3 weeks of age), 163/586 nursery pigs (3-8 weeks of age), 200/586 grow finish pigs (8-25 weeks of age) and 15/586 adult pigs. The age of 129/586 pigs was not given on the submission form. The clinical history of the pigs included no clinical signs (n=2), abortion (n=8), central nervous signs (n=14), respiratory signs (n=315), enteric signs (n=46), mixed respiratory and enteric signs (n=61), systemic disease (n=16), or poor body condition (n=1). No clinical history was given for the remaining 123 cases.

2.1.2. Sample processing. Approximately one gram of lung tissue was minced by scissors, diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM), homogenized by using a Stomacher 80 (Seward Laboratory Systems Inc, Bohemia, NY, USA), and centrifuged at 1500 *g* for 10 min to obtain the supernatant. All samples were stored at -80^{o}C.

2.1.3 Viral DNA extraction. DNA was extracted from 50 µl of each tissue homogenate using the 5×MagMAX^{TM} 96 Viral Isolation Kit (Ambion) according to the manufacturers' instructions on an automated extraction platform (KingFisher Flex; Thermo Fisher Scientific Inc.). DNA was eluted in 50 µl of elution buffer provided in the kit. All DNA extraction procedures included a positive control and a negative control (water) in each run.

2.2. Real-time PCV2 PCR assays

To demonstrate PCV2 DNA in a sample, a Taqman® real-time PCR assay which targets the conserved ORF1 gene of PCV2 and is capable to detect all known PCV2 genotypes including the newly identified PCV2e, without differentiation, was performed as described (Opriessnig et al., 2013). Samples that were ORF1 PCV2 DNA positive were further tested by a multiplex Taqman® real-time PCR which targets the signature motif region within the capsid gene (ORF2) and detects and differentiates PCV2a, PCV2b and PCV2d as described (Opriessnig et al., 2013).To detect the novel PCV2e, a PCV2e specific Taqman® real-time PCR targeting ORF2 was developed which used the following primers PCV2eF: 5-CTCTCCCGCTCCTTTGTATATACTG-3 and PCV2eR: 5-TCCAATATTAAATCTCATCATGTCCAC-3 and the PCV2e probe: CAL Fluor® Orange 560-5'-TAACCTCCACAGTCACACCGCCATCAT-3-BHQ-1. The PCR reactions were carried out in 96-well plates. Each reaction consisted of a total volume of 25 µl, containing 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA), 2.5 µl of sample or standard DNA, 1 µl of 10 µm of each of the two primers, 0.5 µl of 10 µm probe, and 7.5 µl distilled water. Amplification was performed using the ABI7500 Fast Real Time PCR System (Applied Biosystems, Foster City, California, USA) under universal conditions: 2 min at 50°C 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

2.3. PCV2 ORF2 sequencing

To obtain PCV2 ORF2 sequences, all ORF1 PCV2 PCR positive samples

were re-amplified by primers covering the ORF2 gene and were sequenced as described (Opriessnig et al., 2013).

2.4. External obtained PCV2 ORF2 sequences

A total of 372 ORF2 sequences were obtained through the ISU-VDL LIMS data base which corresponded to client case requests, spanned the time frame from January 2014 through April 2016, and included sequences obtained from pigs located in 16 U.S. states (Table 2).

2.5. Sequence alignment and analysis

The sequences were aligned and analyzed with software ClustalW and MEGA5.0 as described (Xiao et al., 2015). The obtained sequences from the tissue set were combined with the ISU-VDL sequences and analyzed.

2.6. Nucleotide sequence GenBank accession numbers

The 455 ORF2 sequences of PCV2 analyzed in the present study have been deposited into GenBank under the accession numbers KU697001-KU697292, KT795288-KT795290, KX098659-KX098782 and KX510050-KX510085.

3. Results

3.1. PCV2 prevalence in randomly selected 2015 lung samples in the USA

Of the 586 lung samples, PCV2 DNA was identified in 23% (135/586) by PCV2 ORF1 real-time PCR. When the samples were categorized by Ct value 3.9% of the samples (23/586) contained high amounts of PCV2 DNA (Ct values < 20.1), 3.6% (21/586) contained medium amounts of PCV2 DNA (Ct values from 20.1 to 25.0), 9.2%% (54/586) contained low amounts of PCV2 DNA (Ct values from 25.1-35.0),

6.3% (37/586) contained insignificant amounts of PCV2 DNA (Ct values from 35.1-40.0) and 77% of the cases (451/586) were negative for PCV2 DNA.

3.2. PCV2 genotypes identified in random lung tissues

Among the 135 ORF1 PCV2 DNA positive samples, 124 were further successfully genotyped by the PCV2 ORF2 differential real-time PCR assay. Specifically, PCV2a was detected in 11.3% of the samples (14/124), PCV2b was detected in 29% of the samples (36/124) and PCV2d was detected in 71.8% of the samples (89/124) (Table 1). Among all 124 samples, 15 samples were co-infected with two different PCV2 genotypes (Table 1).

3.3. Analysis of PCV2 ORF2 sequences

The ORF2 gene of 83 of the 135 PCV2 ORF2 positive samples was successfully sequenced, and 12% (10/83) of the sequences corresponded to PCV2a, 20.5% (17/83) were PCV2b, and 67.5% (56/83) were PCV2d which was in agreement with the multiplex real-time PCR results. PCV2c or PCV2e sequences were not obtained in the samples set. This was further confirmed by a differential real-time PCR assay specific for PCV2e which was negative on all samples.

The genotype distribution of PCV2 genotypes from 2011 through April 2016 based on 598 ORF2 sequences is listed in Table 3. Among the 455 ORF2 sequences obtained in this study (372 external ISU-VDL LIMS sequences and 83 sequences from random lung tissues), 143 previously published ORF2 sequences (Xiao et al., 2015) have also been added for completeness. PCV2c was not detected in the US in the present investigation. Further phylogenetic analysis of the ORF2 sequences

together with reference sequences confirmed the presence of five distinct PCV2 genotypes, and all of the present PCV2d sequences are clustered in the PCV2d-2 clade (Fig. 1). The p-distance of the ORF2 nucleotide sequences of PCV2e with other PCV2 genotypes ranges from 0.15 to 0.17, which is much larger than the PCV2 genotype-definition threshold of 0.035. When the amino acid sequences in the ORF2 gene of the five main PCV2 genotypes were aligned (Fig. 2), PCV2d had four unique amino acid changes and 3 of the 4 amino acids were located in an immunoreactive region. PCV2e had 24 amino acids not present in any of the other genotypes and 12 of these 24 amino acids were located in immunoreactive regions (Fig. 2).

4. Discussion

Retrospective analysis of sequences available through GenBank indicate that PCV2d appeared in 1999 and can be divided into two main groups, PCV2d-1 and PCV2d-2 which contains emerging isolates (Xiao et al., 2015). PCV2d-2 was first recognized in April 2012 in the US (Xiao et al., 2012, 2015). The present data indicate that the prevalence of PCV2d-2 increased from 37.8% in 2012 to 72% in 2016 suggesting that PCV2d-2 has replaced PCV2b and has now become the predominant PCV2 genotype in the USA.

Most of the present samples or cases were from PCV2 vaccinated farms. The majority of the randomly selected lung samples used in this study was obtained from nursery to grow-finish pigs and overall 64.2% had a clinical history of respiratory signs. The great majority of these pigs had been vaccinated with commercial PCV2

vaccines administered at the time weaning as is commonly done in US pig production; however, PCV2 DNA was found in 23% of the investigated cases, with moderate to high amounts in 32.6% (44/135) and with PCV2d-2 identified in 71.8%. This highlights that PCV2d-2 may have some advantage over PCV2a and PCV2b in its ability to replicate in pigs under vaccination pressure.

Among the 44 field cases with moderate to high amounts of PCV2 DNA in the lungs, only 47.7% (22/44) were detected as PCV2 positive by either PCR or PCV2 immunohistochemistry (IHC) tests during the case investigation. PCV2 testing was not requested or done on 41% of these cases (18/44). This suggests that a number of PCVAD cases could remain undiagnosed. In contrast, routine VDL PCR and IHC tests were done on 30.5% of negative or subclinical cases.

The time of origin of PCV2 and the main PCV2 genotypes has been estimated. PCV2 was proposed to originate at the beginning of 20th century (Firth et al., 2009) or between the middle of the 19th and the first half of the 20th century (Franzo et al., 2016). In previous studies, PCV2a was undoubtedly revealed to be the oldest genotype, with the time to most recent common ancestor (TMRCA) estimated to be 1966 (1945-1983) (Firth et al., 2009) or 1964 (1948-1974) (Franzo et al., 2016). The first PCV2 identified in a pig with PCVAD in 1962 was also PCV2a (Jacobsen et al., 2009). For the TMRCA of PCV2b, there is a minor uncertainty as the PCV2b TMRCA has been estimated to be 1989 (1980–1995) (Firth et al., 2009) or 1973 (1952–1996) (Franzo et al., 2016).The earliest PCV2b strain was identified in a PCV2 IHC negative pig in 1979 from Switzerland (Wiederkehr et al., 2009). For PCV2d, the

TMRCA was estimated to be 1986 (1971-1996) based on full genome analysis, which is similar to PCV2b, indicating PCV2d and PCV2b may have bifurcated early and evolved independently (Xiao et al., 2015). However, in a recent study the TMRCA of PCV2d was reported to be much earlier and already in 1958 (1935–1979), even earlier than PCV2b, indicating that PCV2d is older than PCV2b (Franzo et al., 2016). The disagreement in TMRCA for PCV2d has been proposed to result from utilizing different numbers of PCV2d strains for the analysis (Franzo et al., 2016). Up to now, the earliest identified PCV2d sequence in GenBank originated from a pig sample collected in 1999 in Switzerland (Xiao et al., 2015) and no PCV2d sequence has been identified earlier than PCV2b. The conclusion that PCV2d is indeed older than PCV2b is perhaps not sufficiently supported and additional investigations may be needed.

The recent identification of a new PCV2e genotype further supports continuous evolution of PCV2. PCV2e has been present in the US since at least 2006 as determined by a retrospective US investigation (Davies et al., 2016). Interestingly, in this study, from 2013-2016 only nine PCV2e sequences were obtained through submissions and client requests to the ISU-VDL, and no additional PCV2e sequences were identified in the GenBank database during a recent study (Xiao et al., 2015). The obtained PCV2e isolates originated in Iowa (n=7, 2015-2016), Indiana (n=1, 2015) and North Carolina (n=1, 2015). Given that PCV2e has been circulating in the US pig population for at least 10 years its rather low prevalence may indicate that PCV2e is replicating at a low level and perhaps PCV2e also has a lower transmission rate

compared to other PCV2 genotypes.

Further studies are needed to investigate the reasons for the emergence of PCV2d-2 and the importance (if any) of PCV2e. In the US, a genotype shift of 4 years duration from PCV2b to PCV2d-2 appears to have occurred. It is still unclear where the US PCV2d-2 strain originated from and if it truly only entered the US pig population around 2012.

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Figure legends

Fig. 1. The molecular phylogenetic analysis by Neighbor-Joining method. The analysis was based on the present 455 PCV2 complete ORF2 sequences together with 19 reference sequences from GenBank. The evolutionary distances were computed using the p-distance method. All positions containing gaps and missing data were eliminated, and there were a total of 575 positions in the final dataset. The PCV1 sequence were used as an outgroup to root the tree. The analyses were conducted in MEGA6.

Fig. 2. Alignment of representative PCV2a, PCV2b, PCV2c, PCV2d and PCV2e ORF2 amino acid sequence. The grey areas correspond to antibody recognition domains described previously (Trible and Rowland, 2012). The dashed line box shows the location of an immunodominant decoy epitope within the epitope C domain (Trible and Rowland, 2012). Yellow highlighted amino acids are PCV2d specific and green highlighted amino acids are PCV2e specific. "-" represents the same residue as in the top sequence and "." indicates an insertion or a deletion.

Tables

Table 1 Sample origin, number of PCV2 positive samples and PCV2 genotypes identified in 586 lung tissue samples obtained from January to March 2015 in the USA.

State	Number of tissues	PCV2[1]	PCV2 genotype[2]							
			2a	2b	2d	2e	2a+2b	2b+2d	2a+2d	Total
IA	184	46	5	7	27	0	1	4	1	45
NC	73	18	0	2	13	0	0	0	0	15
IL	43	12	1	0	8	0	0	0	0	9
IN	31	3	0	0	2	0	0	0	0	2
MO	21	7	0	0	6	0	1	0	0	7
MN	15	7	0	2	2	0	1	2	0	7
UT	15	6	0	4	2	0	0	0	0	6
NE	13	5	0	3	1	0	0	1	0	5
ND	8	2	0	0	2	0	0	0	0	2
KS	7	1	0	1	0	0	0	0	0	1
CA	6	0	0	0	0	0	0	0	0	0
OK	5	1	0	0	1	0	0	0	0	1
PA	5	1	0	0	0	0	0	0	0	0
VA	6	0	0	0	0	0	0	0	0	0
CO	4	1	0	0	0	0	0	0	0	0
SD	4	0	0	0	0	0	0	0	0	0
AR	3	0	0	0	0	0	0	0	0	0
OH	3	1	0	0	0	0	0	1	0	1
GA	2	2	0	0	2	0	0	0	0	2
SC	2	0	0	0	0	0	0	0	0	0
VT	1	0	0	0	0	0	0	0	0	0
WI	1	1	0	1	0	0	0	0	0	1
Unknown	134	21	4	2	11	0	0	3	0	20
Total	586	135	10	22	77	0	3	11	1	124

[1]Presence of PCV2 DNA was determined by real-time PCR targeting the conserved ORF1 gene (Opriessnig et al., 2013).

[2]Presence and differentiation of PCV2a, PCV2b or PCV2d were determined by differential real-time PCR targeting the ORF2 gene (Opriessnig et al., 2013) and

presence of PCV2e was determined by using a PCV2e specific assay developed in this study.

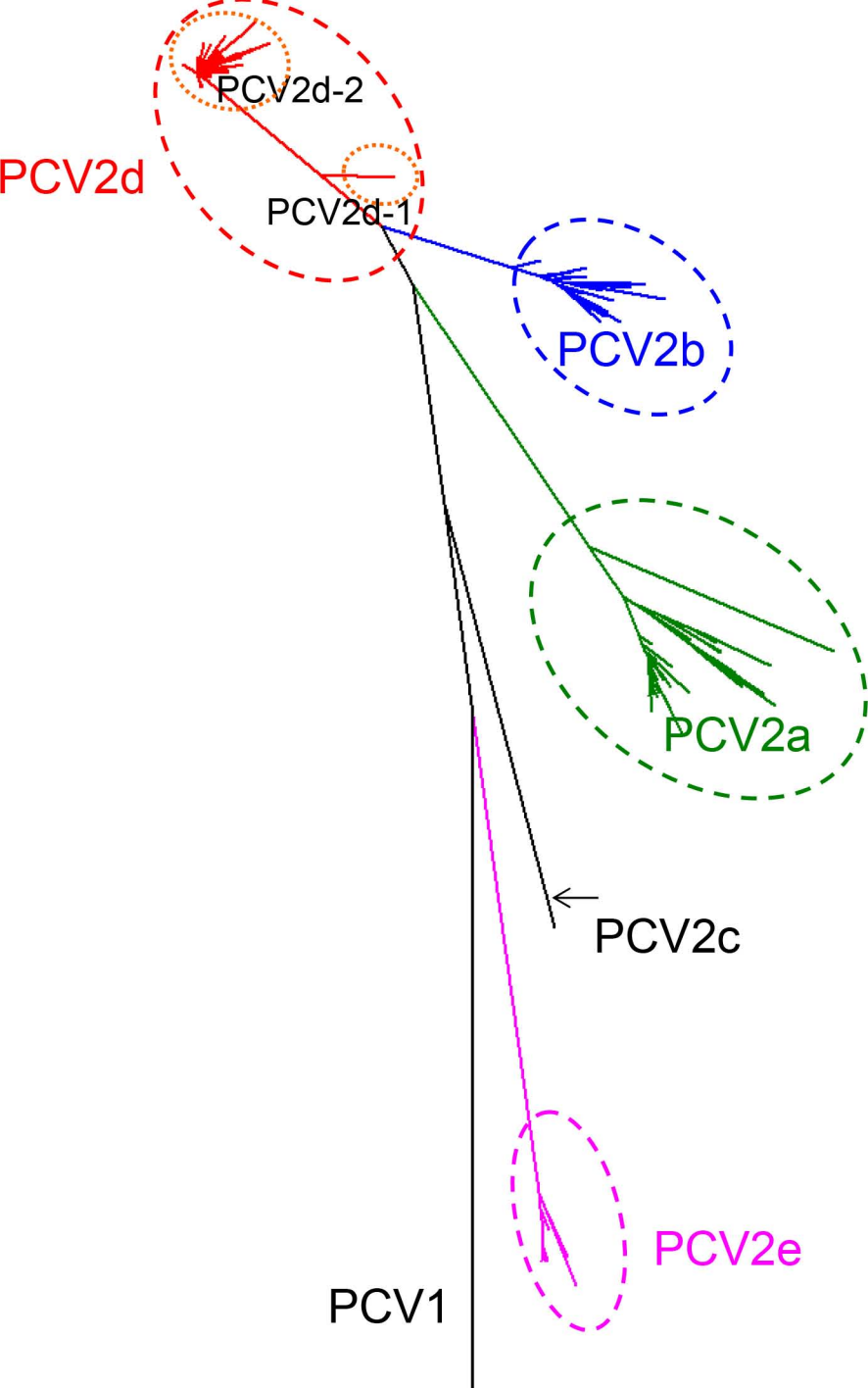
Table 2 Sample origin, number of sequences per state and the PCV2 genotypes identified in 372 ORF2 sequences obtained through the Iowa State University Veterinary Diagnostic Laboratory LIMS system from January 2014 to April 2016.

State	Numbers of sequences included	PCV2 genotype			
		PCV2a	PCV2b	PCV2d	PCV2e
IA	81	19	4	51	7
NC	136	1	11	123	1
MD	17	9	0	8	0
IN	36	7	3	25	1
IL	32	4	2	26	0
MO	11	4	0	7	0
OH	4	0	1	3	0
NE	3	1	2	0	0
WI	3	1	0	2	0
SC	1	0	1	0	0
OK	2	1	0	1	0
MN	6	5	0	1	0
TX	2	1	1	0	0
SD	1	0	0	1	0
KY	1	1	0	0	0
AR	1	0	0	1	0
Unknown	35	10	6	19	0
Total	372	64 (17.2%)	31 (8.3%)	268 (72%)	9 (2.4%)

Table 3 The genotype distribution of PCV2 ORF2 sequences obtained since initial discovery of PCV2d-2 in the US in 2012 through April 2016, including 372 ORF2 sequences obtained through the ISU-VDL LIMS data base and 83 sequences obtained from 2015 lung tissue samples.

	PCV2a	PCV2b	PCV2d-2	PCV2e	Total
2011*	4 (50%)	4 (50%)	0	0	8
2012*	3 (8.1%)	20 (54.1%)	14 (37.8%)	0	37
2013*	11 (11.2%)	51 (53.1%)	36 (36.7%)	0	98
2014	8 (14%)	11 (19.3)	38 (66.7%)	0	57
2015	55 (17.4%)	31 (9.8%)	227 (71.8%)	3 (0.9%)	316
2016 (until April)	11 (13.4%)	6 (7.3%)	59 (72%)	6 (7.3%)	82
Total	92 (15.4%)	123 (20.6%)	374 (62.5%)	9 (1.5%)	598

*Data from Xiao *et al*. 2015.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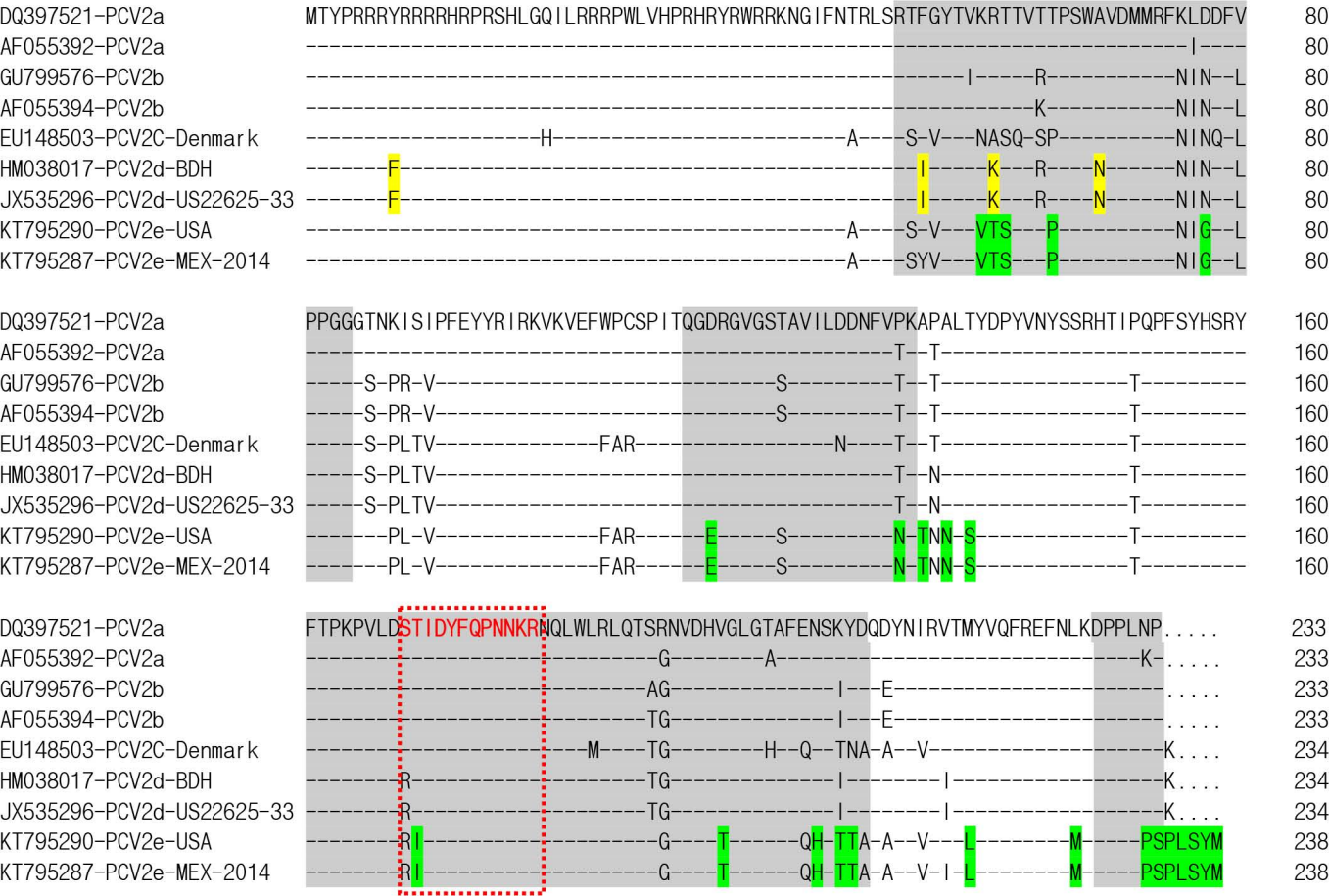
PCV2d
PCV2d-1
PCV2b
PCV2a
PCV2c
PCV2e
PCV1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AF055392-PCV2a 80
GU799576-PCV2b 80
AF055394-PCV2b 80
EU148503-PCV2C-Denmark 80
HM038017-PCV2d-BDH 80
JX535296-PCV2d-US22625-33 80
KT795290-PCV2e-USA 80
KT795287-PCV2e-MEX-2014 80
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AF055392-PCV2a 160
GU799576-PCV2b 160
AF055394-PCV2b 160
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HM038017-PCV2d-BDH 160
JX535296-PCV2d-US22625-33 160
KT795290-PCV2e-USA 160
KT795287-PCV2e-MEX-2014 160
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GU799576-PCV2b 233
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KT795290-PCV2e-USA 238
KT795287-PCV2e-MEX-2014 238